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1. Blumenthal RD, et al. Cancer Res. 1992 Nov 1;52(21):6036-44.
2. Levin LV, et al. Cancer Immunol Immunother. 1987;24(3):202-6.

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Targeted Therapy of Athymic Mice Bearing GW-39 Human Colonic Cancer Micrometastases with ^{131}I-labeled Monoclonal Antibodies[1]

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ABSTRACT

The therapeutic potential of radiolabeled antibodies is usually evaluated in experimental animal models bearing s.c. xenografts. We have established a micrometastatic model of the GW-39 human colonic carcinoma in the nude mouse lung (J. Natl. Cancer Inst., *83:* 627–632, 1991) and presented preliminary findings on the efficacy of a ^{131}I-anti-carcinoembryonic antigen (CEA) antibody in this model. We now extend our observations on the use of radioiodinated labeled monoclonal antibodies (MAbs) to treat multiple small tumor nodules.

Biodistribution and dosimetry analysis was performed for intact and F(ab')$_2$ of NP-4 anti-CEA IgG, Mu-9 anti-colon-specific antigen IgG, isotype-matched irrelevant anti-AFP IgG, and intact MAb 34A anti-lung endothelial IgG antibody. Comparisons were made for rad dose delivered to small s.c. tumors, normal lung, lung with tumor nodules, and isolated tumor nodules. Survival curves were generated for tumor-bearing animals treated 1, 7, or 14 days after tumor cell implantation with these antibodies using the maximal tolerated dose for intact antibodies (275 μCi) and for F(ab')$_2$ fragments (1.2 mCi). The studies established the following observations: (*a*) in contrast to previous results in a bulky tumor model in hamsters, intact antibodies are more therapeutic than MAb fragments for both NP-4 and Mu-9; (*b*) tumor nodule size, even on the microscopic level, affects therapeutic outcome; antibodies were more effective when administered 7 days postimplantation (mean nodule diameter, 150 μm) compared with treatment 14 days postimplantation (mean nodule diameter, 750 μm); (*c*) administration of radioiodinated Mu-9 was exquisitely effective on single avascular tumor cells that had seeded in lung; irrelevant antibody was minimally radiotoxic; (*d*) as in the bulky disease model, the anti-colon-specific antigen p antibody delivers a higher rad dose than the anti-CEA antibody and is significantly more therapeutic in the micrometastasis model; (*e*) a higher affinity anti-CEA antibody (MN-14) recognizing the same epitope on CEA as NP-4 was equally therapeutic; (*f*) the use of MAb directed against the lung endothelium was not as therapeutic as a tumor-associated antibody; and (*g*) all tumor-associated antibodies were more efficacious than administration of the maximal tolerated dose of 5-fluorouracil and leucovorin in this human tumor-xenograft model. These results provide further support for the use of radioimmunotherapy in the handling of minimal disease, probably as part of an adjuvant treatment regimen.

INTRODUCTION

The development of hybridoma technology by Köhler and Milstein (1) created a potential new tool for the diagnosis and therapy of cancer. To determine the usefulness of MAbs[3] directed against human tumor-associated antigens, relevant tumor systems are needed. Human tumors have been transplanted into nude mice and rats, into immunosuppressed mice, and in the hamster cheek pouch. The most commonly used model remains the nude mouse bearing a s.c. tumor (2). Selective tumor targeting of MAbs and growth inhibition by antibodies conjugated with radionuclides, chemotherapeutic agents, or toxins has been shown for a wide array of human xenografts grown s.c. as bulky tumors in athymic nude mice (3–5).

Mathematical analysis of radiolabeled antibody uptake into human tumor xenografts has revealed an inverse correlation between tumor mass and tumor uptake (6). This relationship between antibody uptake and tumor size has also been demonstrated in patients (7). As a result, small tumors can be eradicated, while larger tumors exhibit a growth reduction, but not a cure (3, 8). Therefore, small primary tumor lesions or small multiple metastatic sites should be considered to be the most suitable targets for RAIT.

Most cancers do not present clinically as bulky s.c. sites. Since location of a tumor may influence the physiology of the tumor and, thereby, the ability to target that tumor with antibody conjugates (9), it is important to develop tumor models that reflect both the appropriate size and the site of tumor growth that are compatible with the clinical situation. We have presented preliminary results on the development of a micrometastatic lung model of the human colonic tumor, GW-39 (10). Injection of a cell suspension of GW-39 i.v. results in ~40–80 tumor nodules (50–200 μm in diameter within 7 days) throughout the lungs, and all animals die of extensive tumor involvement within 5–10 weeks. Using this model, we have demonstrated prolonged survival and a reduction in the number of viable tumor colonies histologically with suboptimal doses of ^{131}I-labeled antibody (150 μCi) against CEA. In this paper, we further evaluate the ability of RAIT to eradicate microscopic disease. The efficacy of tumor-specific antibodies (NP-4 anti-CEA IgG and Mu-9 anti-CSAp) IgG and an irrelevant antibody (AFP-7–31) IgG, administered as an intact antibody or as an F(ab')$_2$ fragment at their MTD, have been evaluated.

In addition to testing antitumor antibodies, the use of an organ-specific antibody was examined. Kennel *et al.* (12) recently described the development of an anti-endothelial monoclonal antibody with enhanced specificity for lung endothelium. The targeting and therapeutic potential of this antibody labeled with ^{131}I were evaluated in the GW-39 experimental lung metastasis model. Finally, a comparison of these procedures was made to conventional chemotherapy using 5-fluorouracil with leucovorin.

MATERIALS AND METHODS

Animal Model. GW-39 human colonic tumors (112) were maintained by serial propagation of s.c. tumors in 6–7-week-old female athymic *nu/nu* mice (Harlan Sprague Dawley). Tumors were excised, minced in sterile 0.9% NaCl, and passed through a 40 mesh screen to

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[3] The abbreviations used are: MAb, monoclonal antibody; CEA, carcinoembryonic antigen; CSAp, colon-specific antigen p; MTD, maximal tolerated dose; 5-FUra, 5-fluorouracil; RAIT, radioimmunotherapy; i.a., intraarterial; i.c., intracecal; i.s., intrasplenic.

produce a 10% suspension (w/v). Tumors were initiated by injecting 30 μl of the cell suspension into the caudal tail vein.

Radioantibody Preparation. NP-4 anti-CEA IgG and Mu-9 anti-CSAp IgG have been described previously (3, 13). These monoclonal antibodies were purified from mouse ascites using protein A and ion-exchange chromatography over S-Sepharose (Pharmacia, Piscataway, NJ). An irrelevant isotype-matched antibody, anti-AFP IgG MAb (designated AFP-7-31), was obtained from Immunomedics, Newark, NJ. Monoclonal antibody 34-A, a rat IgG2a recognizing mouse lung endothelium, and an irrelevant isotype-matched antibody (MAb 14-A) were purified from ascitic fluid by ammonium sulfate precipitation and DEAE-cellulose chromatography (12).

$F(ab')_2$ fragments were prepared by pepsin digestion in 0.1 M sodium citrate buffer, pH 3.5, at 37°C for 45–90 min. The reaction was stopped by elevating the pH to 7.0. The preparation was desalted on a column of Fractogel TSK HW-50F (EM Science, Gibbstown, NJ) and fractionated by ion-exchange chromatography on Q-Sepharose that had been equilibrated with Tris-HCl buffer, pH 7.5. The $F(ab')_2$ was collected and concentrated by YM-30 ultrafiltration (Amicon, Danvers, MA) and dialyzed against 0.04 M phosphate-buffered saline, pH 7.4.

Antibodies were radioiodinated by the chloramine-T method (14). Free iodine was separated from antibody-bound iodine by passage over a PD-10 column (Pharmacia) that had been equilibrated with 0.04 M phosphate-buffered saline (0.04 M phosphate, 0.15 M NaCl, and 0.02% NaN_3), pH 7.4, containing 1% human serum albumin. Size exclusion high-pressure liquid chromatography of each radioantibody preparation was performed to monitor the molecular size of the labeled antibody and to measure the unbound iodine content. No sample had more than 4% unbound iodine and 2% aggregation when used. Immunoreactivity on CEA- or CSAp-extracted preparations bound to Affi-Gel (Bio-Rad, Richmond, CA) was also determined for NP-4 preparations (70–80%) and the Mu-9 preparations (78–88%).

Biodistribution Studies. Two groups of animals were used for these studies, one group with GW-39 lung tumor with a small (~0.1-g) s.c. tumor and the other with only a small s.c. tumor. The percentage of radioantibody uptake in normal lung, lung containing 3-week-old GW-39 nodules, several tumor nodules dissected from the lung, and small (~0.1g) s.c. GW-39 tumors was determined by injecting 10 μCi (6 μg) of ^{131}I-labeled specific antibody and 5 μCi (1.5 μg) of ^{125}I-labeled irrelevant antibody. Groups of 5 animals were sacrificed 1, 3, 7, and 14 days after intact IgG injection or 1, 3, and 7 days after $F(ab')_2$ injection. The percentage of injected dose/g was recorded. All data were corrected for physical decay of the isotopes and the backscatter of ^{131}I into the ^{125}I channel.

Dosimetry Calculations. Biodistribution data were used to generate time-activity curves for the calculation of radiation doses to tumor and normal tissues, as described previously (15). Data for normal tissues were fit to an exponential curve and integrated over all time. The integral of tumor activity was computed trapezoidally by assuming activity equals zero at time zero. Since conventional medical internal radiation dose tables provide S-factors for tissues larger than 1.0 g, S-factors were generated to account for the smaller size of the animal tissues (16).

Tumor Therapy Studies. At specified times post-tumor implantation (1, 7, or 14 days) animals were given i.v. injections of the MTD (the highest possible dose resulting in no animal death, *i.e.*, 275 μCi of an IgG and 1.2 mCi of an $F(ab')_2$) of radioiodinated antibodies. 5-FUra (150 mg/kg) and leucovorin (90 mg/kg) given i.v. 2 h prior to 5-FUra were also used at the MTD. The MTD for the radiolabeled antibodies and 5-FUra were determined empirically in tumor-bearing animals. Animals were weighed on the day that radioantibody was administered and weekly thereafter. Survival was monitored weekly and daily once the animal lost >20–25% of its starting body weight. Immediately after death, lungs were removed, fixed in formalin, sectioned, and stained with hematoxylin/eosin to assess viability of individual tumor colonies and to evaluate if the cause of death was tumor related. Colonies that had undergone complete karyolysis were considered nonviable. This approach was selected instead of adoptive transfer because it is uncertain how many viable cells within a predominantly nonviable colony are necessary to induce growth of a new tumor upon transfer. In addition, the number of parameters assessed, the number of animals per group, and the number of colonies that had to be evaluated per animal prevented the latter approach from being feasible.

Statistical Evaluation. The Kaplan-Meier product-limit (17) method was used to estimate the survival function of each treatment group. Comparison of any two groups was carried out using the log rank test (18). Because of the limited number of animals per group (n = 10–20), sensitivity of test results is an issue; hence the results should be interpreted with care. In the comparison of Mu-9 IgG for the 7-day-old and 14-day-old models, there was only 1 death among the 10 animals in the 7-day group. For this comparison, it was not feasible to perform a log rank test; instead, the survival probability of the IgG 7-day group was fixed at 0.9 for the entire study period and then compared with the survival probability of the IgG 14-day group at a given time. An approximate one-sample test based on an assumed normal distribution was used here.

Microautoradiography. Tumors were removed 72 h after injection of 100 μCi of ^{125}I-Mu-9 or ^{125}I-MAb 34A and fixed in cold acid-ethanol. Serial 5-μm sections were cut from paraffin-embedded sections and rehydrated. Slides for microautoradiography were dipped into an aqueous (1:1) dilution of Kodak NTB-2 emulsion. Following a 7-day exposure in light-tight boxes at 4°C, the slides were processed using Kodak Dektol developer and GBX fixer at 15–19°C and counterstained in hematoxylin.

RESULTS

By dose escalation, the MTD for the ^{131}I-labeled intact antibody and the $F(ab')_2$ fragment was determined to be 275 μCi and 1.2 mCi, respectively. These doses were used in all survival therapy studies. Fig. 1 illustrates the survival of mice with varying tumor burdens, either 7-day implants with 40–80 nodules averaging 125 μm in diameter or 14-day implants with nodules averaging 750 μm in diameter treated with NP-4 anti-CEA or the nonspecific anti-AFP antibody. Untreated mice die within 7–10 weeks post-tumor implantation (mean survival, 7.3 weeks). The nonspecific antibody fragment and intact antibody prolong mean survival by 3 and 5 weeks, respectively, for both the 7- and 14-day models. In contrast, NP-4 IgG increased mean survival by 20.3 weeks in 7-day implants and by 11.3 weeks in 14-day implants compared with untreated mice ($P <$

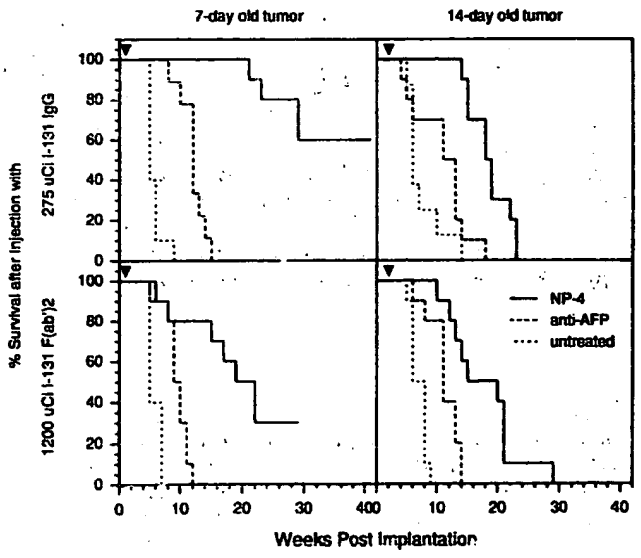

Fig. 1. Survival curves of nude mice bearing either 7- or 14-day-old GW-39 lung nodules and treated with the maximal tolerated dose of NP-4 anti-CEA or irrelevant antibody anti-AFP-7-31 IgG (275 μCi) or $F(ab')_2$ fragment (1200 μCi). Lung nodules were initiated by caudal vein injection of 30 μl of a 10% GW-39 suspension. All groups contained 10 animals. ▼, time when the radioantibody was administered.

0.0001 for both the 7d and the 14d models). The improvement in survival was clearly reduced when antibody was administered at 14 days instead of at 7 days post-tumor cell implantation. The results for NP-4 $F(ab')_2$ are similar; mean survival is increased by 11.7 weeks if administered 7 days after implantation and by 8.5 weeks, when administered 14 days after implantation ($P < 0.001$ and $P < 0.004$, respectively). For the 7-day-old model, the IgG was more efficacious than the $F(ab')_2$ ($P = 0.04$), but there was no significant difference in survival of mice with 14-day-old tumors treated with the 2 forms of NP-4. This antibody-specific increase in survival results from a dose of 1717 rads from the intact antibody and 550 rads from the $F(ab')_2$ to the tumor nodules (Table 1). For both antibody forms, the rad dose is less than what is delivered to a small (0.1-g) s.c. tumor by these two antibody forms and almost 2-fold greater than the rad dose delivered by the irrelevant anti-AFP antibody (Table 2). In fact, specific uptake of NP-4 in the isolated nodules or whole tumor-bearing lung was not discernible until day 14 postadministration, whereas specific uptake (tumor/blood ratio >1) in the s.c. tumor was evident by day 7.

The importance of antibody affinity for tumor targeting and therapy has been a debated topic, since MAb affinity may influence accretion, retention, intratumor penetration, and complexation with circulating antigen. A comparison of NP-4 anti-CEA with MN-14 anti-CEA, a MAb recognizing the same epitope on CEA, but with a 10-fold higher affinity, revealed no significant enhancement of animal survival using the GW-39 micrometastasis model (Fig. 2), even though MN-14 accretion is greater than NP-4 accretion in small (0.1-g) s.c. tumors (results not shown).

Both intact antibody and fragments of Mu-9 anti-CSAp have been shown previously to be more therapeutic than NP-4 anti-CEA in the bulky GW-39 hamster cheek pouch model (13). Survival data shown in Fig. 3 indicate that this relationship also exists for GW-39 lung nodules. Thirty-six weeks after tumor implantation, only 1 of 10 animals succumbed to tumor burden in the 7-day treatment group with either the intact antibody or the $F(ab')_2$ ($P < 0.001$ compared with the anti-AFP MAb). Once again, the larger tumor nodules in the 14-day treatment group were less responsive; 50% mortality in mice treated with Mu-9 IgG (mean survival, 31.8 weeks) and 60% mortality following Mu-9 $F(ab')_2$ after 37 weeks (mean survival, 14 weeks). The increase in survival for Mu-9-treated mice compared to the irrelevant IgG or $F(ab')_2$ is highly significant ($P < 0.0001$). For the 7-day model, there was no significant difference between the Mu-9 IgG and the Mu-9 $F(ab')_2$ treated groups. However, the IgG was more efficacious in the 14-day-old model ($P < 0.001$). Histological evaluation of lungs taken from mice that survived 37 weeks revealed a lack of viable tumor cells in 55% of the colonies, while 45% of the colonies contained a few nucleated cells (Fig. 4). The potential for repopulation by these "viable" cells is unknown. In all cases, the size of the nodule at their time of death from animals given the specific antibody was much smaller (diameter, 0.75–1.5 mm) than from animals evaluated at the time of death after treatment with the irrelevant antibody (diameter, 3.0–4.0 mm). Unlike NP-4, specific targeting of Mu-9 could be appreciated by either counting the tumor nodules or the whole tumor-bearing lung. Biodistribution and dosimetric analyses for Mu-9 indicated much higher rad doses to tumor nodule than from the NP-4 MAb; 4,629 rads from the IgG and 4481 rads from the fragment (Table 3) compared with 1717 rads from the IgG and 550 rads from the $F(ab')_2$.

Since Mu-9 was so effective on the smaller 7-day lung nodules, we evaluated its efficacy when administered 24 h after cell implantation, when cells are known to have seeded in the lung, but have not established a vascular network yet. After 36 weeks, 100% of mice treated with the specific antibody were alive with no evidence of tumor growth in the lungs (Fig. 5). In contrast, 90% of the mice treated with an equal dose of the irrelevant antibody did not survive beyond 12 weeks. Although the control anti-AFP MAb increased median survival by 3 weeks, this effect was attributed to nonspecific radiotoxicity. The therapeutic advantage of the specific MAb with the 1-day-old model is similar to what was observed with the 7-day-old model and may suggest a potential role of a specific antibody carrier as an adjuvant following surgical debulking.

In the next study, we compared the therapeutic potential of organ-specific targeting of a radionuclide with direct tumor immunotargeting. The rationale for this type of study is based on the hypothesis that destruction of blood vessels feeding tumor colonies will affect the viability of the colony. In addition, recent results demonstrating antigen heterogeneity among tumor cells in a given host and between primary and metastatic tumors suggests a need for a panel of antibodies to be used for targeting to work effectively. Organ-specific targeting, such as with MAb 34A, the lung anti-endothelial antibody (12) that targets a common epitope in normal tissue, the expression of which will probably not be as variable as the tumor antigens, will require only 1 antibody.

Microdistribution of both tumor-directed Mu-9 and lung-directed 34A are shown in Fig. 6. Mu-9 (Fig. 6*A*) distributes around each individual nodule, with minimal penetration, as has been observed in larger tumors (19). MAb 34A distributes homogeneously throughout the lung but cannot be found in or around tumor colonies (Fig. 6*B*). Uptake of MAb 34A was very high at the earliest time point (24 h; Table 4) but unlike the

Table 1 *Biodistribution and dosimetry: NP-4 anti-CEA*

Tissue	NP-4 IgG[a] (% ID[b]/g)				NP-4 $F(ab')_2$[c] (% ID/g)			
	24 h	168 h	336 h	Rads[d]	24 h	72 h	168 h	Rads[e]
s.c. tumor[f]	14.9 ± 3.9[g]	24.8 ± 6.8	27.8 ± 3.3	6457	6.3 ± 0.7	4.4 ± 1.0	0.6 ± 0.3	2257
Normal lung[h]	8.6 ± 4.1	4.9 ± 1.6	2.5 ± 0.8	1328	1.6 ± 0.3	0.4 ± 0.05	0.02 ± 0.01	412
Tumor-bearing lung	7.1 ± 2.3	7.6 ± 0.9	5.2 ± 1.3	1996	2.4 ± 0.9	0.5 ± 0.1	0.2 ± 0.2	566
Tumor nodules	8.2 ± 2.9	4.8 ± 0.6	5.5 ± 1.7	1717	2.3 ± 0.5	0.6 ± 0.2	0.2 ± 0.1	550
Blood	16.8 ± 2.5	7.6 ± 2.1	3.5 ± 0.8	2764	3.8 ± 0.7	0.7 ± 0.06	0.03 ± 0.01	895

[a] s.c. tumors weighed 0.172 ± 0.083, 0.133 ± 0.056, and 0.091 ± 0.037 g on days 1, 7, and 14, respectively. Nodules weighed 0.010 ± 0.007, 0.023 ± 0.005, 0.049 ± 0.014 g on day 1, 7, and 14, respectively.
[b] ID, injected dose.
[c] s.c. tumors weighed 0.072 ± 0.022 and 0.219 ± 0.195 g on days 1 and 7, respectively. Nodules weighed 0.037 ± 0.010 and 0.059 ± 0.029 g on days 1 and 7, respectively.
[d] The dose to the tissues is based on the MTD of 275 μCi.
[e] The dose to the tissues is based on the MTD of 1.2 mCi.
[f] MAb uptake in s.c. tumors was not influenced by the presence of tumor in lung.
[g] Mean ± SD ($N = 5$).
[h] Normal lung is from animals that only have a s.c. tumor.

Table 2 *Biodistribution and dosimetry: AFP-7-31 anti-AFP*

Tissue	AFP-7-31 IgG[a] (% ID[b]/g)				AFP-7-31 F(ab')$_2$[c] (% ID/g)		
	24 h	168 h	336 h	Rads[d]	24 h	168 h	Rads[e]
s.c. tumor	6.8 ± 1.2[f]	4.4 ± 0.8	3.4 ± 1.1	1294	1.3 ± 0.3	0.04 ± 0.02	644
Normal lung	7.5 ± 3.1	4.9 ± 1.4	3.3 ± 1.2	1162	1.1 ± 0.3	0.02 ± 0.01	288
Tumor-bearing lung	6.2 ± 2.0	5.9 ± 0.7	6.0 ± 1.5	1281	1.5 ± 0.4	0.04 ± 0.01	566
Tumor nodules	6.7 ± 2.3	3.2 ± 0.7	5.3 ± 1.3	993	1.1 ± 0.4	0.08 ± 0.06	535
Blood	12.6 ± 0.5	10.6 ± 1.7	6.3 ± 2.0	2331	1.2 ± 0.4	0.02 ± 0.01	330

[a] s.c. tumors weighed 0.172 ± 0.083, 0.133 ± 0.056, and 0.091 ± 0.037 g on days 1, 7, and 14, respectively. Nodules weighed 0.010 ± 0.007, 0.023 ± 0.005, 0.049 ± 0.014 g on days 1, 7, and 14, respectively.
[b] ID, injected dose.
[c] s.c. tumors weighed 0.357 ± 0.098 and 0.648 ± 0.320 g on days 1 and 7, respectively. Nodules weighed 0.008 ± 0.002 and 0.016 ± 0.002 g on days 1 and 7, respectively.
[d] The dose to tissues is based on the MTD of 275 μCi.
[e] The dose to tissues is based on the MTD of 1.2 mCi.
[f] Mean ± SD (N = 5).

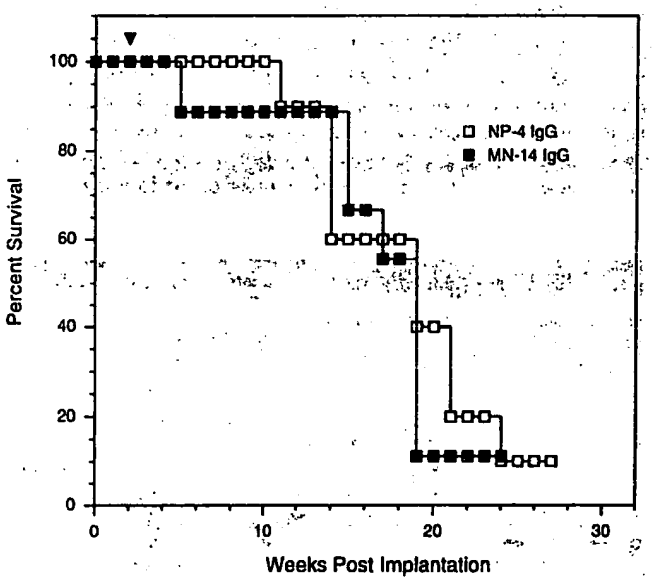

Fig. 2. Comparison of mouse survival with 14-day-old GW-39 lung micrometastases treated with the MTD (275 μCi) of either lower affinity ^{131}I-NP-4 IgG or higher affinity ^{131}I-MN-14 IgG (n = 10). ▼, time when the radioantibody was administered.

antitumor antibodies decreased rapidly over time. Administration of MAb 34A 14 days after tumor cell implantation resulted in an increase in mean survival from 7.3 to 13.3 weeks (Fig. 7). However, this effect appears to be a result of nonspecific radiotoxicity, since an isotype-matched irrelevant antibody (14-A) was equally effective, even though the rad dose delivered to tumor-bearing lung was 17,367 for MAb 34A and only 934 for MAb 14A (Table 4). Although the rad dose to isolated nodules seems quite high (7449 rads), it is probably an artifact of small amounts of normal lung tissue in the sample, since autoradiographic results show no grains within the nodules from this MAb. Histological evaluation of lungs taken from these animals revealed 5% of the colonies to be completely nonviable, while the remaining colonies were large and fused with a high proportion of viable cells, suggesting that death was due to tumor progression. Some lung samples appeared to be thickened or brittle; however, specific pulmonary tests were not performed to gauge the possible damage to pulmonary function, if any. Furthermore, histological evaluation was performed 11–14 weeks after the delivered radioantibody dose and 99% of the radiation dose is delivered during the first 2 weeks, allowing time for tissue repair to occur. It was interesting to note that in 2 studies in which MAb 34A was used, approximately 25% of the experimental animals developed pronounced metastases at various sites within their bodies (ovary, bone, spleen, liver, stomach, and spinal cord), resulting in tremors and hind leg paralysis in several animals. In only 2 samples from >150 untreated mice and >400 mice treated with either tumor-specific or irrelevant MAb could GW-39 be found in sites outside of the lung. The development of metastases after MAb 34A might be explained by disruption of the lung vasculature, thereby permitting the release of tumor cells into the circulation for seeding elsewhere. Alternatively, the extension in survival time, however small, may have provided sufficient time to permit growth of cells seeded at the time of tumor implantation that have otherwise gone undetected when animals die of lung disease earlier. This observation of extrapulmonary sites following therapy with ^{131}I-MAb 34A will be expanded to more animals to establish its significance.

Systemic or hepatic i.a. 5-FUra with either leucovorin or levamisole are the most common methods of treatment of advanced colorectal cancer (20, 21). We evaluated the efficacy of 5-FUra in combination with leucovorin administered at the MTD in mice with 14-day-old GW-39 lung nodules. Fig. 8 demonstrates a 3-week extension of life span with a single treatment and no improvement in life span using fractionated dosing of 5-FUra (total dose delivered using 5 equal daily doses). 5-FUra and leucovorin resulted in the same 2–3-week extension of survival in 7-day-old and 1-day-old lung implants (data not shown). Therefore, for this particular model, radioantibody

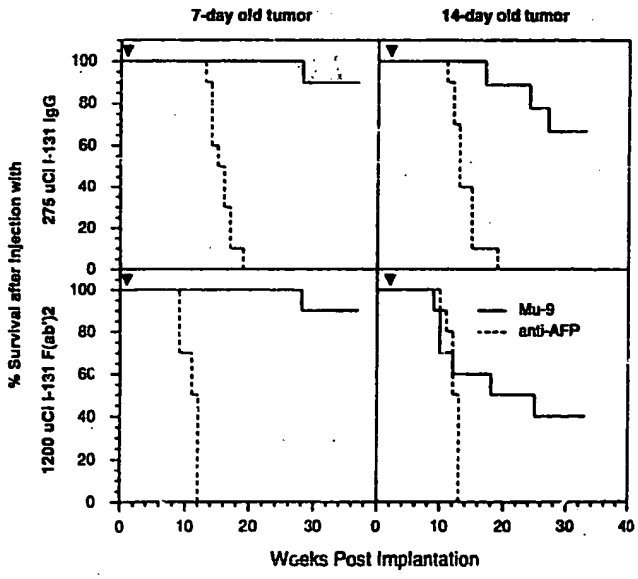

Fig. 3. Survival curves of nude mice bearing either 7- or 14-day-old GW-39 lung nodules and treated with the maximal tolerated dose of Mu-9 anti-CSAp or irrelevant antibody anti-AFP-7–31 IgG or F(ab')$_2$ fragment. Seven-day-old transplants had 16 animals/group and 14-day-old transplants had 20 animals/group. ▼, time when the radioantibody was administered.

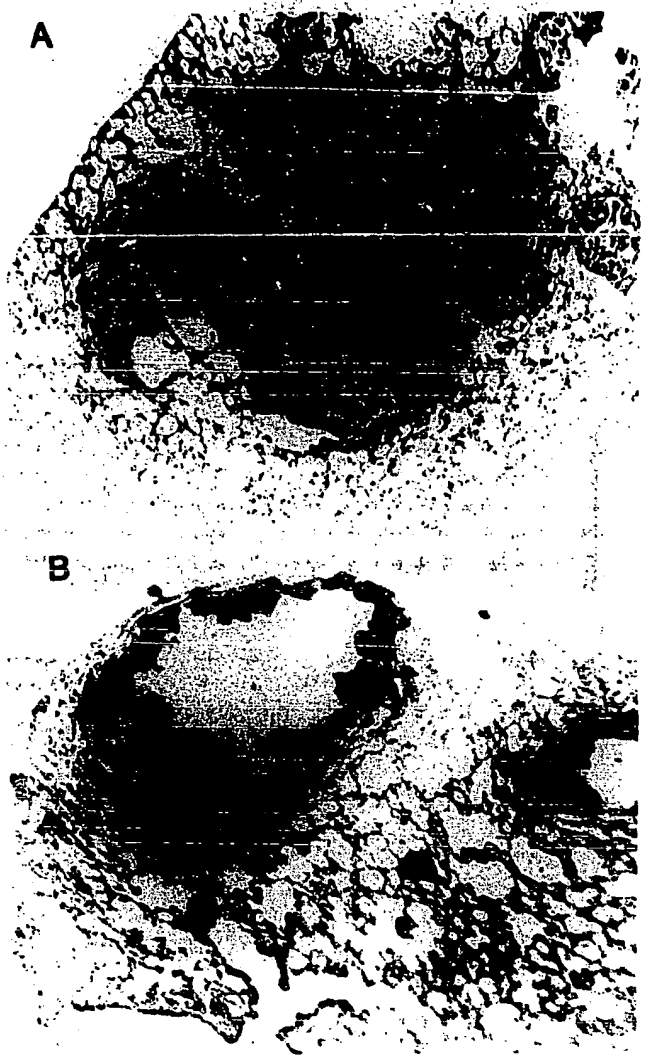

Fig. 4. Sections of untreated viable GW-39 lung nodules (*A*) and nonviable nodules posttherapy with the maximal tolerated dose of ^{131}I-Mu-9 IgG (*B*). H & E, × 40.

therapy was significantly more efficacious than the combination of 5-FUra and leucovorin.

The survival data for all studies are summarized in Table 5. The probability of survival has been calculated at 3 time points post-implantation of the tumor (12, 16, and 24 weeks) for each therapeutic. As is clearly indicated, the best treatment in this human colon xenograft model is Mu-9 IgG and the least effective treatment is 5-FUra with leucovorin.

DISCUSSION

Animal modeling of human carcinomas is often restricted to the s.c. site. However, this site may not be an accurate reflection of the visceral sites of growth observed in patients. In order to understand the biology of tumor metastases and to evaluate therapeutic modalities for these sites, human xenograft models have been used. Several investigators have reported the results of treatments with a variety of antitumor agents (immunomodulators, drugs, and radiopharmaceuticals) in lung and liver metastasis models of melanomas, lymphomas, and sarcomas (22–25).

Animal modeling of human colon carcinoma has been performed by i.c. and i.s. implantation of colon carcinoma cells in athymic mice to produce multiple liver metastases (26, 27). Other reports have shown that some highly aggressive tumors can metastasize following s.c. foot pad or i.v. injection (28, 29). Since the i.v. route is a relatively easy mode of implantation in contrast to the abdominal surgery required for the development of liver metastases, and since hematogenous metastases to the lungs is known to occur in colorectal cancer patients, we selected the i.v.-induced lung model to establish basic principles on the efficacy of RAIT in a metastatic model. Injection of a suspension of GW-39 colonic cancer cells i.v. results in ~40–80 nodules throughout the lung and all mice die of extensive tumor involvement within 5–10 weeks (10).

Among the challenges facing investigators is the selection of an appropriate MAb and optimal antibody form. The choice of each of these variables may not be the same for tumors of varying (*a*) histopathology, (*b*) size, or (*c*) site of growth. The evaluation of these variables in different animal models might aid in establishing principles of clinical relevance, as has been the case in the past with the use of murine models for evaluating other therapeutic modalities (30, 31).

Mathematical analysis has suggested that antibodies with higher affinity may result in higher MAb accretion and a prolonged retention time in the tumor (32, 33). However, these improvements afforded by higher antibody affinity may be counterbalanced by reduced antibody penetration within the tumor and increased complexation with circulating antigen, which might be expected to interfere with tumor targeting. Preclinical studies with the two anti-CEA MAbs used in these studies (NP-4 and MN-14) that recognize the same class-III CEA epitope but differ in affinity by 10-fold suggest better targeting with the higher-affinity MN-14 MAb.[4] Clinical results with the same antibodies demonstrate excellent tumor targeting with MN-14, even in patients with elevated serum CEA (34), although it is not yet certain whether there is a clear clinical advantage over NP-4. The therapeutic benefit of MN-14 compared with NP-4 in our micrometastasis model was similar. This is particularly important, since the limitations of higher affinity antibodies previously mentioned are not a concern in our study, *i.e.*: (*a*) tumor penetration would not be expected to be a serious concern on the micrometastatic level; and (*b*) clinical data do not support interference of circulating antigen with targeting of anti-CEA MAbs. A comparison of the therapeutic potential of each MAb in a bulky tumor model (large s.c. tumor or large single liver mass) of GW-39 remains to be determined. In another model system, immunohistochemical and radiolocalization studies with second generation anti-TAG-72 antibodies showed greater reactivity and improved antitumor effects with the higher-affinity antibody (CC49) with carcinoma cells and/or tumor-associated mucin in a majority of tissue samples than with the lower-affinity B72.3 MAb (35).

Selection of the appropriate form of antibody for RAIT involves several considerations. The kinetics of distribution and uptake are different for intact MAb and F(ab')$_2$ fragments. Intact antibodies have a longer circulating half-life, are retained for a longer time by tumor tissue, and can thereby deliver a higher radiation dose. Dosimetry calculations for administration of the MTD of NP-4 IgG and F(ab')$_2$ in s.c. tumors or in tumor nodules of similar size reveal that if equitoxic amounts of radioactivity are administered, the intact antibody can deliver almost 3 times the radiation dose to the tumor than the fragment (Table 1). For Mu-9, the rad dose to tumor is greater for

[4] H. J. Hansen, E. S. Newman, R. Grebenau, R. M. Sharkey, and D. M. Goldenberg. Second generation anti-carcinoembryonic antigen monoclonal antibodies. Preclinical characterization. submitted for publication.

Table 3 *Biodistribution and dosimetry: Mu-9 anti-CSAp*

Tissue	Mu-9 IgG[a] (% ID[b]/g)				Mu-9 F(ab')$_2$[c] (% ID/g)		
	24 h	168 h	336 h	Rads[d]	24 h	168 h	Rads[e]
s.c. tumor	27.9 ± 3.4[f]	111.8 ± 22.0	111.4 ± 43	14,785	10.7 ± 2.2	5.6 ± 1.7	6,522
Normal lung	8.3 ± 1.4	4.0 ± 1.0	1.5 ± 0.6	1,159	2.0 ± 0.6	0.03 ± 0.01	535
Tumor-bearing lung	29.1 ± 5.1	22.0 ± 10.4	13.7 ± 3.9	4,969	12.0 ± 1.9	3.2 ± 1.2	5,078
Tumor nodules	15.5 ± 2.2	31.2 ± 12.8	11.8 ± 2.3	4,629	10.3 ± 2.5	3.0 ± 1.0	4,481
Blood	19.7 ± 1.5	7.9 ± 1.4	1.5 ± 0.4	2,670	3.3 ± 0.9	0.02 ± 0.01	85!

[a] s.c. tumors weighed 0.149 ± 0.030, 0.162 ± 0.099, and 0.201 ± 0.058 g on days 1, 7, and 14, respectively. Nodules weighed 0.013 ± 0.002, 0.017 ± 0.009, and 0.050 ± 0.007 g on days 1, 7, and 14, respectively.
[b] ID, injected dose.
[c] s.c. tumors weighed 0.357 ± 0.098 and 0.648 ± 0.320 g on days 1 and 7, respectively. Nodules weighed 0.008 ± 0.002 and 0.016 ± 0.002 g on days 1 and 7, respectively.
[d] The dose to tissues is based on the MTD of 275 μCi.
[e] The dose to tissues is based on the MTD of 1.2 mCi.
[f] Mean ± SD (N = 5).

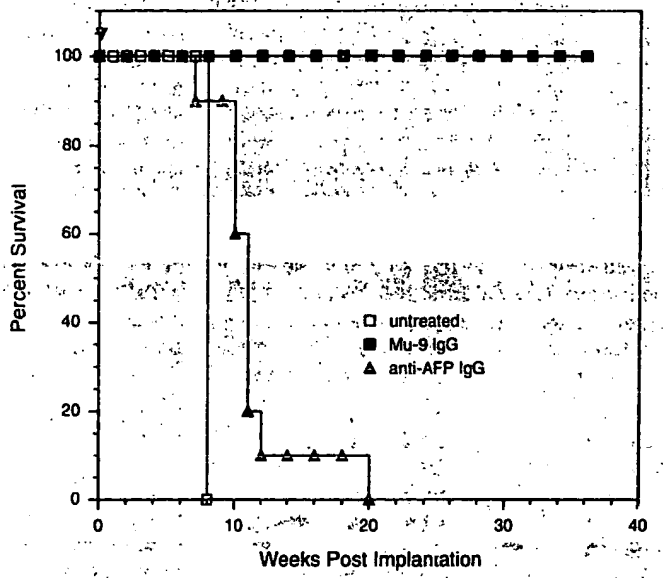

Fig. 5. Survival curve for nude mice treated with RAIT 1 day after tumor cell transplantation. Mice were treated with 275 μCi ^{131}I-Mu-9 IgG or anti-AFP IgG (n = 10). ▼, time when the radioantibody was administered.

intact antibody in s.c. tumors; however, the rad dose is similar for both forms in lung nodules (Table 3). This finding can be explained by the difference in the kinetics of uptake of each form of Mu-9 in s.c. tumors and in lung nodules, the intact antibody exhibiting high accretion and prolonged retention in the s.c. mass. Another consideration might be the ability of the antibody to permeate out of vessels feeding tumor tissue and penetrate throughout the tumor. Although lower molecular weight fragments are likely to permeate vessels better, the avascular nature of these micrometastatic sites (limited to 1 to 2 vessels on the perimeter of the nodule) may limit the permeability advantage of the fragment. In several models, fragments have been shown to penetrate more rapidly and more deeply into solid tumors than intact antibody (36, 37). However, in our animal model, the topographic pattern of antibody distribution within micrometastatic tumors grown in lung or liver was similar for both the intact antibody and the F(ab')$_2$ fragment (38). Furthermore, with very small masses, one would not expect antibody penetration to be a critical factor. In previous therapy studies using bulky (0.4 cm^3) GW-39 tumors grown in the hamster cheek pouch, we have observed better tumor therapy, even though the delivered rad dose was lower, with antibody fragments than with intact antibody (13). A direct comparison of intact antibody and F(ab')$_2$ fragments at the MTD in a bulky nude mouse s.c. model suggests similar growth retardation by both antibody forms. With the GW-39 micrometastasis model, intact IgG is more efficacious that F(ab')$_2$ fragments for both NP-4 and Mu-9 at 2 sizes of nodules (7 and 14 days postim plantation). The differences in therapeutic potential of the two forms of antibody could not be explained by nonspecific radiation effects, since survival was similar for both the anti-AFP IgG and F(ab')$_2$. Thus, the choice of optimal antibody form may in part be dependent on the size and location of the mass to be treated, intact antibodies being more efficacious in microscopic disease and fragments being equally effective or more advantageous in bulky disease.

The dosimetric calculations is this study have demonstrated that the maximal tolerated dose for all intact antibodies results in a blood dose of 2300–2800 rads while the MTD for F(ab')$_2$ fragments results in a blood dose of 600–900 rads. This observation highlights the importance of determining both the total

Fig. 6. Microautoradiography of ^{131}I-Mu-9 IgG around GW-39 lung nodules (*A*) and 34A IgG anti-endothelial MAb distribution throughout normal lung (*B*). Mice were given injections 14 days post-cell implantation with 100 μCi of ^{131}I-MAb and sacrificed 3 days later. H & E, × 40.

Table 4 *Biodistribution and dosimetry: 34A anti-lung endothelium and 14A isotype matched irrelevant antibody*

Tissue	34-A IgG[a] (% ID[b]/g)				14A IgG[c] (% ID/g)			
	24 h	168 h	336 h	Rads[d]	24 h	168 h	336 h	Rads[e]
s.c. tumor	1.6 ± 0.2[f]	0.7 ± 0.06	0.3 ± 0.1	871	5.7 ± 0.9	0.4 ± 0.06	0.1 ± 0.06	596
Normal lung	290.9 ± 21.8	66.6 ± 9.6	6.7 ± 3.5	27,013	12.9 ± 5.2	0.1 ± 0.3	0.2 ± 0.13	408
Tumor-bearing lung	181.5 ± 31.1	46.2 ± 11.5	7.7 ± 7.8	17,367	6.1 ± 1.9	0.5 ± 0.8	0.3 ± 0.13	446
Tumor nodules	95.4 ± 70.4	13.7 ± 8.1	2.4 ± 2.6	7,449	8.6 ± 3.9	0.7 ± 0.7	0.2 ± 0.14	524
Blood	3.0 ± 0.9	1.3 ± 0.34	0.5 ± 0.3	1,370	10.1 ± 1.5	0.7 ± 0.1	0.3 ± 0.1	742

[a] s.c. tumors weighed 0.100 ± 0.026, 0.192 ± 0.053, and 0.204 ± 0.065 g on days 1, 7, and 14, respectively. Nodules weighed 0.015 ± 0.004, 0.019 ± 0.006, and 0.066 ± 0.013 g on days 1, 7, and 14, respectively.
[b] ID, injected dose.
[c] s.c. tumors weighed 0.100 ± 0.026, 0.192 ± 0.053, and 0.204 ± 0.065 g on days 1, 7, and 14, respectively. Nodules weighed 0.015 ± 0.004, 0.019 ± 0.006, and 0.066 ± 0.013 g on days 1, 7, and 14, respectively.
[d] The dose to tissues is based on a dose of 275 μCi.
[e] The dose to tissues is based on a dose of 275 μCi.
[f] Mean ± SD ($N = 5$).

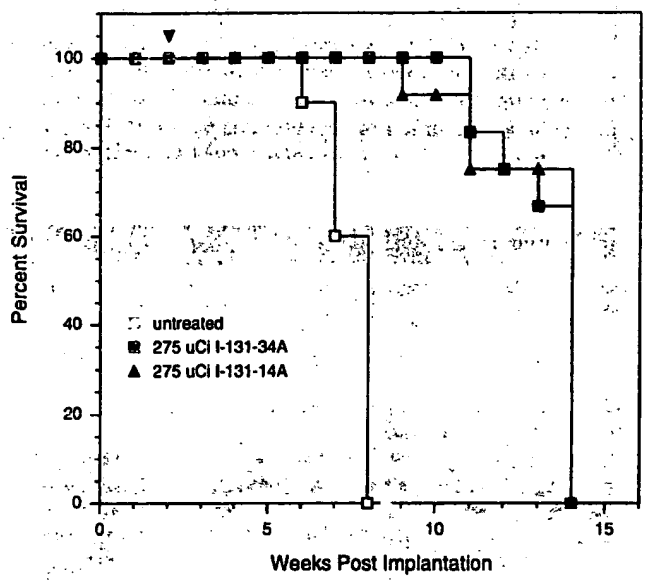

Fig. 7. Survival curve for nude mice with 14-day-old lung transplants treated with the anti-endothelial antibody (34A) or an isotype-matched irrelevant antibody (14A) at a matched dose of 275 μCi as was used for the tumor specific antibodies. ▼, time when the radioantibody was administered.

rad dose delivered and the dose rate of delivery. Both the IgG and $F(ab')_2$ deliver about 200 rads over the first 24 h and 300–400 rads from 24–48 h post-administration to the blood. Even though the dose for the $F(ab')_2$ trails off after 48 h, while the intact antibody continues to provide a substantial dose for the next few days, the maximal toxicity has already been reached. Therefore, the early part of the dose *versus* time posttreatment profile is critical.

It is interesting to note that the rad dose delivered by the irrelevant antibody was the same in both the s.c. tumor and the lung nodules, whereas both specific antibodies (NP-4 and Mu-9) and both forms of antibody deliver a higher rad dose to s.c. tumors than to tumor nodules (Table 2). This is somewhat surprising since smaller tumors are known to accrete more antibody as a percentage of injected dose/g than larger tumors (39). This difference probably cannot be explained by differences in the vascular network supporting the two sites of growth because of equal dosing by the irrelevant antibody. Rather, the difference in uptake may be a function of differences in antigen content in the s.c. site *versus* the lung site.

A comparison of the survival of mice bearing GW-39 lung colonies after treatment with an anti-CEA or an anti-CSAp MAb with mice given MAb 34A provides strong evidence for the advantage of a tumor-targeting antibody for therapy. Although we and others have shown greater growth inhibition with a radiolabeled tumor-associated antibody than an isotype-matched irrelevant antibody (3, 40, 41), the studies reported here are the first demonstration known to us of the therapeutic potential of an antibody directed against tumor cells *versus* an antibody that recognizes the visceral site where the tumor is growing, *i.e.*, the capillary endothelial cells of the lung (12). Microautoradiography clearly demonstrates that Mu-9 is found surrounding tumor nodules and not in normal lung, and MAb 34A is found throughout normal lung tissue but not in tumor nodules. Although MAb 34A has been used successfully to target liposomes to mouse lung (42), and MAb 34A delivers a 6-fold higher rad dose to tumor-bearing lung than our best tumor-associated MAb, Mu-9, the therapy results of the organ-specific MAb were disappointing. MAb 34A was no more effective at increasing mouse survival than MAb 14A, the isotype-matched irrelevant antibody that delivers less than 8% of the rad dose to tumor-bearing lung that is delivered by 34 Å. These results suggest that more intimacy of the radionuclide to the tumor tissue is required. However, MAb 34A labeled with ^{90}Y might be more efficacious than ^{131}I-labeled MAb because more of the radiation dose from antibody in the normal lung will reach neighboring tumor nodules.

Further support for the potential role of RAIT as an adjuvant therapy, following surgical debulking of one or more large primary site(s), comes from our results with the 1-day-old tumor

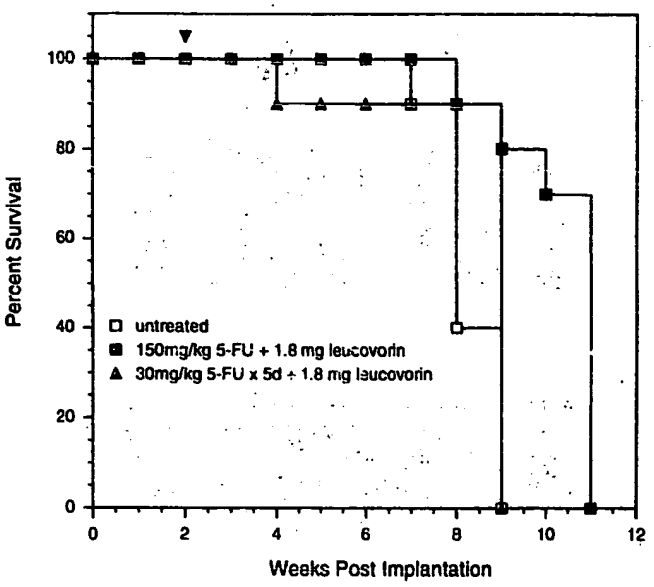

Fig. 8. Survival curve for nude mice with 7-day-old lung xenografts treated with a single maximal tolerated dose (150 mg/kg) of 5-fluorouracil (*5-FU*) plus 1.8 mg leucovorin or a fractionated dose schedule of 30 mg/kg 5-FUra plus 360 μg leucovorin for 5 days. ▼, time when the radioantibody was administered.

Table 5 *Estimated probability of survival posttherapy*

Days post-tumor transplantation	Treatment	Probability of survival		
		12 wk	16 wk	24 wk
1	Untreated	0.0	0.0	0.0
1	^{131}I-AFP IgG	0.1 (0.1)[a]	0.0	0.0
1	^{131}I-Mu-9 IgG	1.0	1.0	1.0
7	Untreated	0.0	0.0	0.0
7	^{131}I-AFP IgG	0.3 (0.2)	0.0	0.0
7	^{131}I-NP-4 IgG	1.0	1.0	0.8 (0.1)
7	^{131}I-Mu-9 IgG	1.0	1.0	1.0
7	^{131}I-AFP F(ab')$_2$	0.0	0.0	0.0
7	^{131}I-NP-4 F(ab')$_2$	0.8 (0.1)	0.7 (0.2)	0.3 (0.2)
7	^{131}I-Mu-9 F(ab')$_2$	1.0	1.0	1.0
14	Untreated	0.0	0.0	0.0
14	^{131}I-AFP IgG	0.7 (0.2)	0.1 (0.1)	0.0
14	^{131}I-NP-4 IgG	1.0	0.7 (0.2)	[b]
14	^{131}I-Mu-9 IgG	1.0	0.8 (0.1)	0.3 (0.2)
14	^{131}I-AFP F(ab')$_2$	0.4 (0.2)	0.0	0.0
14	^{131}I-NP-4 F(ab')$_2$	0.8 (0.1)	0.5 (0.2)	[b]
14	^{131}I-Mu-9 F(ab')$_2$	0.6 (0.1)	0.4 (0.1)	0.3 (0.1)
14	^{131}I-34A IgG	0.8 (0.1)	0.0	0.0
14	^{131}I-14A IgG	0.8 (0.1)	0.0	0.0
14	5-FUra + leucovorin	0.0	0.0	0.0

[a] Numbers in parentheses, SE of estimated probability.
[b] Not evaluable because follow-up time is less than 24 weeks.

model. Using ^{51}Cr-labeled cells, we have observed that 24 h after i.v. injection of cells, some colon cancer cells have seeded in the lung (^{51}Cr cannot be found in other tissues, but at earlier time points, ^{51}Cr can be detected in blood, liver, and kidney).[5] This 1-day-old transplant serves as a good model for the ability of tumor-associated antibody conjugates to seek, find, and destroy tumor cells, even under avascular conditions. The use of ^{131}I-Mu-9 in this model resulted in 100% survival after 36 weeks and an absence of any identifiable lung colonies on gross examination of lung tissue, in contrast to a maximum 8-week survival of untreated mice and a maximum 12-week survival of mice treated with the irrelevant ^{131}I-AFP antibody.

To date, the most common form of treatment for colorectal cancer is chemotherapy with 5-FUra. Although the benefit to the 5-year survival of patients treated with 5-FUra alone has not been substantial, the combination of 5-FUra with either leucovorin or levamisole has improved responsiveness in patients with metastatic cancer and has reduced the recurrence of stage C colorectal cancer (20, 21). The variability in responsiveness to 5-FUra is great between different colonic cancer lines (43), and since GW-39 grows poorly *in vitro*, the only cytotoxicity data for 5-FUra for this line come from *in vivo* studies using the hamster cheek pouch model. This drug resulted in ~10% growth inhibition in contrast to a 30–40% reduction in growth by other drugs such as actinomycin C and Proresid (44). We used the GW-39 to compare the therapeutic efficacy of RAIT *versus* 5-FUra plus leucovorin at the maximal tolerated dose of each treatment modality. Whereas the median survival of mice with 14-day-old nodules was prolonged by 30 weeks with NP-4 anti-CEA treatment, chemotherapy resulted in a 3-week increase in survival. RAIT has also been shown to be superior to 5-FUra therapy by Schlom *et al.* (45) in the LS174T xenograft model. We are currently expanding these studies and comparing the antitumor effects of anti-CEA radioantibody therapy and 5-FUra plus leucovorin therapy in several colonic tumors of varying histopathology, CEA expression, and *in vitro* responsiveness to 5-FUra.

In summary, the results presented here demonstrate the efficacy of radioiodinated antibodies administered at the MTD in microscopic nodules of a human colonic carcinoma xenograft grown in a visceral area. The data highlight several points: (*a*) intact antibodies are more therapeutic than F(ab')$_2$ fragments, an observation that differs from our previous data with a bulky s.c. tumor model; (*b*) tumor nodule size, even on the microscopic level, affects therapeutic outcome, and radioantibodies are exquisitely effective on single avascular tumor cells; (*c*) antibodies recognizing different antigens (CEA and CSAp) exhibit differences in therapeutic benefit for the same size nodules; (*d*) an antibody directed against the endothelial tissue of the site of tumor growth does not affect therapeutic outcome; and (*e*) all tumor-associated radioantibodies are more efficacious than the use of 5-FUra and leucovorin in this animal xenograft model. These results taken together continue to support the use of RAIT in an adjuvant setting.

[5] Unpublished results.

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